



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : G01N 33/68, 33/539	A1	(11) International Publication Number: WO 97/18476 (43) International Publication Date: 22 May 1997 (22.05.97)
(21) International Application Number: PCT/AU96/00711 (22) International Filing Date: 8 November 1996 (08.11.96) (30) Priority Data: PN 6493 10 November 1995 (10.11.95) AU (71) Applicants (for all designated States except US): THE UNIVERSITY OF WESTERN AUSTRALIA [AU/AU]; Nedlands, Perth, W.A. 6009 (AU). RAMSAY HEALTH CARE PTY. LTD. [AU/AU]; 154 Pacific Highway, Greenwich, NSW 2065 (AU). (72) Inventor; and (75) Inventor/Applicant (for US only): MARTINS, Ralph, Nigel, Walter [AU/AU]; 18 Watsonia Street, Maddington, W.A. 6109 (AU). (74) Agents: HARWOOD, Errol, John et al.; Wray & Associates, 239 Adelaide Terrace, Perth, W.A. 6000 (AU).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>
(54) Title: A METHOD FOR DIAGNOSING ALZHEIMER'S DISEASE (57) Abstract <p>A method for detecting Alzheimer's disease in a patient, said method comprising the steps of: (i) selecting from a patient, suspected of having Alzheimer's disease, a fluid sample containing secreted amyloid precursor protein; (ii) reacting the fluid sample proteins with a binding substance specific for either the β-amyloid peptide or the amyloid precursor protein or both; and (iii) assaying for the presence of a higher than normal quantity of secreted amyloid precursor protein with an average molecular weight of greater than about 130 kilodaltons, compared to patients negative for Alzheimer's disease.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgyzstan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	KZ	Kazakhstan	SG	Singapore
CH	Switzerland	LJ	Liechtenstein	SI	Slovenia
CI	Côte d'Ivoire	LK	Sri Lanka	SK	Slovakia
CM	Cameroon	LR	Liberia	SN	Senegal
CN	China	LT	Lithuania	SZ	Swaziland
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	LV	Latvia	TG	Togo
DE	Germany	MC	Monaco	TJ	Tajikistan
DK	Denmark	MD	Republic of Moldova	TT	Trinidad and Tobago
EE	Estonia	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	UG	Uganda
FI	Finland	MN	Mongolia	US	United States of America
FR	France	MR	Mauritania	UZ	Uzbekistan
GA	Gabon			VN	Viet Nam

A Method for diagnosing Alzheimer's disease

The present invention relates to a method for diagnosing Alzheimer's disease in a patient by identifying the presence of and or the relative abundance of a form of secreted amyloid precursor protein which has an average molecular weight of
5 greater than about 130 kilodaltons.

Alzheimer's disease (AD) is a common form of cerebral degeneration which leads to dementia. It is characterised by the deposition of amyloid in intracellular and extracellular compartments of the cerebral cortex. At present the disease can only be conclusively diagnosed at death following postmortem examination
10 of the brain for the presence of amyloid deposits.

AD is characterised by the deposition of amyloid in amyloid plaque cores and as congophilic angiopathy which are localised in the extracellular compartments of the brain and as neurofibrillary tangles within neurons. The major protein component of extraneuronal amyloid deposits is a small peptide termed beta-
15 amyloid ($A\beta$) which is proteolytically derived from its much larger parent molecule termed the amyloid precursor protein (APP).

APP is a transmembrane protein which consists of a family of different protein isoforms that are generated by alternate splicing of the 19 exons of the APP gene. Three major forms of APP are APP695, APP751 and APP770. APP695
20 differs from the other two major forms in that it lacks exon 7 which codes for a 57-amino acid domain with homology to Kunitz-type protease inhibitors.

Cells of haematopoietic origin have been found to splice out exon 15, resulting in 3 additional forms of APP. These 3 additional forms collectively termed leucocyte derived APP (L-APP) differ from the original APP695, APP751 and
25 APP770 isoforms, by lacking 18 amino acids, which are coded by exon 15. These L-APP isoforms consisting of 677, 733 and 752 residues correspond to the exon 15 containing isoforms APP695, APP751 and APP770, respectively.

The role of APP in the immune response has been investigated by a number of researchers. It has been observed that resting peripheral mononuclear blood cells secrete very low levels of APP but in response to known mitogens that stimulate T-cell proliferation, these cells secrete abundant levels of APP. *In vivo* activated T-cells which over express APP have been observed in the disease polymyositis, an inflammatory myopathy. The APP rich T-cells, in polymyositis, have been shown to be invasive front cells that penetrate the basal lamina of the endomysial tube and displace the muscle fiber. Both CD8+ (cytotoxic T-cells) and CD4+ (helper T-cells) cells also express abundant APP suggesting that APP plays a more general role in the immune system.

The major forms of secreted APP from stimulated T-lymphocytes contain high molecular weight isoforms (125-130 kilodaltons (kD)) derived from L-APP. A high molecular weight form of secreted APP has been reported to be produced in both early-onset and late-onset familial AD (FAD) from lymphoblastoid cell lines (Matsumoto, A. and Fujiwana, Y. (1991) Biochem. Biophys. Res Commun., 175, 367-365). However, this higher molecular form of 120 kD has been found to be a complex containing a protease and a 65 kD fragment of APP which lacks the beta amyloidogenic sequence.

The processing of APP is thought to play a key role in the pathogenesis of AD. Strong evidence in favour of APP playing a central role in AD came from research which initially identified mutations in the APP gene located on chromosome 21, in some families with the disease. The first APP mutation linked to AD was shown to occur at codon 717 of the APP gene where valine was substituted for isoleucine. Transfected cell lines over-expressing this mutant APP gene have increased levels of extended forms of the amyloidogenic proteolytic product beta amyloid (A β) compared to cell lines over expressing the normal APP gene.

There is now evidence to suggest that APP is cleaved from membranes, as part of its neurotrophic function (ie. it is capable of enhancing the survival or

outgrowths of nerve processes). Cleavage is thought to occur at a particular site within the A β sequence. Abnormal cleavage of APP appears to lead to A β production which in turn is thought to form proteinaceous amyloid plaque cores; a principle molecular component of AD.

- 5 Various genetic tests have been developed which seek to identify individuals at risk of developing AD. These methodologies rely upon the detection of specific mutations in known genes. Moreover, they predict which individuals would develop AD but cannot determine whether the patient is developing or has the disease. Furthermore, there are yet no genetic tests for the majority of AD
- 10 patient. The present invention seeks to alleviate some of the problem with the prior art.

Summary of the invention

The present invention provides a method for assaying for Alzheimer's disease in a patient said method comprising the steps of:

- 15 (i) selecting from a patient, suspected of having Alzheimer's disease, a fluid sample containing secreted amyloid precursor protein;
- (ii) reacting the fluid sample proteins with a binding substance specific for either the β -amyloid peptide or the amyloid precursor protein or both; and
- 20 (iii) assaying for the presence of a higher than normal quantity of secreted amyloid precursor protein with an average molecular weight of greater than about 130 kilodaltons.

While the present invention relates to the assaying of secreted amyloid precursor proteins in fluid samples, the relative concentration of secreted

25 amyloid precursor proteins in fluid is often very low. The specificity of the method may, however, be increased by selecting and culturing *in vitro*, a cellular source of tissue that is known to produce secreted amyloid precursor proteins. The culture supernatant can then be assayed for the presence of secreted

amyloid precursor proteins with an average molecular weight of greater than about 130 kilodaltons.

More particularly, the invention consists in a method for detecting Alzheimer's disease in a human said method comprising the steps of:

- 5 (i) selecting a biological sample from a patient suspected of having alzheimer's disease, wherein said sample is isolated from a cell source which in alzheimer's disease patients is known to produce secreted amyloid precursor protein.;
- (ii) growing the biological sample selected in step (i) under suitable
10 cell culture conditions for sufficient time to allow amyloid precursor protein expression and secretion into the culture supernatant;
- (iii) reacting, the culture supernatant, with a binding substance specific for either the β -amyloid peptide or the amyloid precursor protein or both; and
- 15 (iv) assaying the proteins selected in step (iii) for the presence of a higher than normal quantity of protein with an average molecular weight of greater than about 130 kilodaltons.

The subject invention may be used to detect long forms of secreted APP (i.e. forms of secreted APP of greater than 130 kilodaltons in molecular weight) from
20 a range of biological sources. Preferably, the secreted APPs have an average molecular weight of approximately 130 to 140 kD. Increased production of secreted APP's of a molecular weight of 130 kD to 140 kD have not previously been identified in high abundance in AD patients. Detection and quantitation of such proteins provides a means for identifying and quantifying the onset of AD.

25 In patients suffering from AD, there is a higher concentration of secreted APPs with a molecular weight greater than 130 kD compared to AD negative patients. To distinguish AD positive from AD negative patients the method of the present invention should be carried out with parallel control experiments using fluid or tissue samples from known AD negative patients. The identification of a

difference between the control and sample sources is taken as being indicative of the onset of AD. If visual differences can not be readily identified between the control and sample sources the results of the method should be quantified. Methods for quantifying the amounts of protein in an electrophoretic band are
5 known in the art. Preferably, the results are quantified using densitometry.

Examples of fluid sources from in which secreted APPs are found in humans include blood plasma and cerebral spinal fluid. Examples of cellular sources which are known to secrete APPs include, lymphoblastoid cells and skin fibroblasts. Preferably, the cellular source material is lymphoblastoid cells.

- 10 Binding substances suitable for use in the invention may be of any form provided that they are capable of selecting either the β -amyloid peptide or the amyloid precursor protein or both proteins. Preferably, the binding substances individually or in combination recognise part or all of the first 24 amino acids of the A β peptide or the C-terminus of APP. For example, the binding substance
15 may be an antibody which recognises part or all of one or more of the following sequences: amino acids 17 through 24 of the A β peptide; amino acids 1 through 16 of the A β peptide; or the C-terminus of APP.

- When the binding substances interact with the secreted APPs they form a complex and can be readily selected. The selected secreted APPs can then be
20 assayed for and examined by molecular weight analysis. Methods of molecular weight analysis are known in the art and include, for example, gel exclusion chromatography, gel filtrations, ultra centrifugation, SDS PAGE electrophoresis analysis, western blots etc.

- In one embodiment of the present invention, proteins obtained from either the
25 fluid sample or the culture supernatant may be concentrated and/or precipitated before being incubated with the binding substance specific. Methods of concentrating and precipitating proteins are well known in the art and include, for example, centrifugation, altering the conditions in the sample to precipitate the proteins and like methods.

In one particularly preferred embodiment of the invention lymphoblastoid cells are selected as the biological sample and are grown under suitable cell culture conditions for sufficient time to allow amyloid precursor protein expression and release into the culture supernatant.

- 5 In addition to assaying for secreted APPs in fluid the method of the invention may also be modified to accommodate a qualitative comparison of the difference between secreted APPs in fluid and APPs in the cellular tissue. In such a method the cellular tissue grown in cell culture is separated from the culture supernatant. Preferably the cells are grown in the presence of a biosynthetic
- 10 label which labels produced proteins. The cells are then disrupted using methods known in the art. The lysate and culture supernatant is then reacted separately with binding substances capable of selecting either part or all of the first 24 amino acids of the A β peptide or the C-terminus of APP. The selected proteins samples are then analysed for APPs of 130 to 140 kD molecular weight.
- 15 It will be appreciated that the method of the present invention may have general application for identifying various forms of AD where abnormalities in the cleavage of A β result in the production of a secreted APPs of 130 to 140 kD molecular weight. In particular, however, the present method provides a means for assaying for familial AD (FAD) caused by abnormal processing of APP
- 20 resulting in a secreted form of APP with a molecular weight of 130 to 140 kD.

Detailed description of the invention

- The molecular weight of processed secreted APP in AD negative patients is typically in the order of about 100 to 110 kD molecular weight. The 130 to 140 kD high molecular weight form of secreted APP is typically observed in affected
- 25 individuals with a missense mutation at codon 717 in the APP gene (i.e. it results from a point mutation in exon 17 of APP which results in a substitution of isoleucine for a valine at residue 717 (APP770)). The same high molecular weight form of secreted APP is also observed in early onset FAD lymphoblastoid cell lines containing a defect in chromosome 14. Both of these defects have

- 7 -

been linked to early onset FAD. While little is presently known about this high molecular weight form of APP it is expected that the protein may consist of either the entire or substantially all of the amylogenic precursor protein before it is proteolytically cleaved.

- 5 Mutations on the APP gene located on chromosome 21 account for only a small percentage of FAD families. It is now known that most forms of FAD result from genetic defects in other chromosomes in addition to chromosome 21. Recent research suggests that mutations on other chromosomes may have an impact on the processing of the APP gene which in turn may be responsible for the
- 10 generation of abnormal APP products. One such defect is on chromosome 14 where a mutation in the presenilin 1 (PS1) gene (formerly known as the S182 gene) may influence abnormal APP processing. Evidence has been accumulated to show that mutations in the PS1 gene are associated with increased levels of A β in blood, skin fibroblasts and the brains of FAD patients.
- 15 Binding substances suitable for use in the invention include antibodies, antibody fragments or antibody complexes which recognise at least an epitope within the A β peptide such as an epitope within part or all of the first 24 amino acids of the A β peptide and/or the C-terminus of the APP. Antibodies suitable for use in the invention may be either monoclonal or polyclonal antibodies. In a particularly
- 20 preferred form the invention the monoclonal antibodies are used in the method which are capable of detecting part or all of the first 16 amino acids of the A β peptide or part or all of amino acids 17 to 24 in the A β peptide.

It will be appreciated that the binding substances used in the method of the invention may be employed in a variety of ways to identify the presence of

25 secreted APPs of a molecular weight 130 to 140 kD. For example, the binding substances may be used to selectively extract secreted APPs for a fluid sample. Alternatively, they may be used to selectively extract APP from the total protein content of, for example, disrupted cells. In either case the selected proteins are preferably dissociated from the binding substance and then analysed on a gel.

Detection of the protein may then be achieved by detection means known in the art such as, by using radioactive or fluorescent labelled probes (eg labelled antibodies) or by silver staining techniques.

Methodologies that may be used to identify the long forms of secreted APP may include, for example, enzyme linked immunoabsorbant assays (ELISA), radioimmunoassays (RIA), or immunoblot assays. When using such methodologies, the proteins in the biological sample are first separated according to their molecular weight differences. The band(s) corresponding to greater than 130 kD is then identified. The presence of secreted APPs with a molecular weight greater than 130 kD may be identified using such substances as labelled binding substances (e.g. using labelled antibodies that are specific for the binding substances), or by other method known in the art which facilitates such detection. Labels which may be coupled to the binding substances (i.e. directed against the binding substance) are those known in the art and include but are not limited to enzymes, radionucleotides, fluourogenic and chromogenic substrates, cofactors and biotin-avidin, colloidal gold and magnetic particles.

Techniques especially useful for large scale clinical screening of suspected AD patients include ELISA and radioimmunoassays. Such techniques are preferred for their speed, and their ability to test numerous samples simultaneously and ease of automation.

Protocols upon which ELISA assays may be based include, for example, competition assays, direct reaction assays and sandwich type assays. In ELISA assays binding substances or antibodies directed against APP's generally are preferable coated into wells in, for example, a microtitre tray where an immunological complex forms if abnormal APP's or APP's generally are present in the sample. A signal generating means may be added to detect complex formation. A detectable signal is produced if abnormal APP's are present in the sample.

In a preferred embodiment of the invention, lymphoblastoid cells are selected as the biological sample. The selection of lymphoblastoid cells from human patients

may be achieved by suitable methods known in the art. The cell are then preferably immortalised and grown in the presence of a biosynthetic label which is capable of labelling proteins.

- One method that is particularly preferred for identifying longer forms of secreted APP's involves growing familial AD, sample and control lymphoblastoid cells in the presence of labelled amino acid(s) under suitable conditions to allow the label to be incorporated into proteins that are synthesised by the cells. At a suitable time after incubation the culture media is then separated from the cells and the cells are washed to remove excess label. The cells are then solubilised to release all of their cytoplasmic and membrane proteins. APP's of a molecular weight of 130 to 140 kD may then be precipitated from the cell lysate and the culture medium using the binding substances described above. Detection of any of the APP's may be achieved using electrophoretic gel or by employing standard detection means known in the art.
- Immortalisation may be achieved by fusing the lymphoblastoid cells to a variety of stabilised cell lines to maintain stability. Alternatively, the lymphoblastoid cells may be infected with transforming viruses (e.g. Sendai virus or Epstein Barr virus), or transfected with transforming genes to create permanent or semi-permanent cell lines. In addition, the lymphoblastoid cells may be fused to established cell lines. Methods of cell fusion are known in the art and may include, for example, the use of fusion agents such as polyethylene glycol. Preferably, the cells are immortalised using Epstein Barr virus.

- Conditions suitable for culturing, for example, lymphoblastoid cells are generally known in the art. Typically, the procedure involves incubating cells in, for example, RPMI 1640 medium containing 5 to 30% foetal calf serum at about 30 to 40°C in the presence of 1 to 10% CO₂. Preferably, the cells are incubated in RPMI 1640 medium containing 20% foetal calf serum at 37°C in the presence of 5% CO₂.

- 10 -

The time period for incubation of the biological sample will vary depending on the environmental conditions in which the cell sample is grown. The cells should, however, be grown for a suitable time period to allow APP production and secretion into the cell culture supernatant. Preferably the cells are

5 incubated under such conditions in the presence of a radioactive amino acid for at least 10 hours, preferably for 16 to 72 hours and most preferably 24 to 48 hours to permit optimal APP detection.

Methods of labelling of amino acids are subject to a great deal of variation. Labels that may be used include: fluorescent, chemiluminescent, radioactive or

10 dye molecules. Preferably, the lymphoblastoid cells are incubated in the presence of at least a radioactively labelled amino acid(s) for at least 1 hr. More preferably they are incubated in the presence of the labelled amino acid(s) for 1 to 48 hrs. Between 4 to 24 hrs and more specifically 5 to 16 hrs are the most desirable time periods for incubating with the label.

15 It will be appreciated that the method of the present invention may also be made available in the form of a kit. Preferably, the kit comprises a binding substance specific for either the β -amyloid peptide or the amyloid precursor protein or both and instructions consistent with the method of the present invention. In addition, the kit may also comprise one or more of suitable media for growing a biological

20 sample, solutions for disrupting a cellular sample, binding substances specific for part or all of the first 24 amino acid residues of β -amyloid peptide and or a binding substance specific for the C-terminus of APP, one or more solutions or media suitable for detecting high molecular weight (ie 130 to 140 kD) APP.

The present invention will now be described by reference to the following non-

25 limiting figures and examples. It will be understood that all of the parameters prescribed in the examples are given as indicative only, and that parameters outside these limits may also provide useful results.

In the Figures:

Figure 1 depicts a family tree of an Australian family with a mutation in the APP gene at codon 717 where the amino acid valine is substituted with isoleucine. Those family members whose lymphocytes were immortalised by infection with Epstein-Barr virus in accordance with the following methods and used for the study of APP metabolism are indicated by an associated asterisk.

Figure 2 is a photographic representation of an electrophoretic gel visualisation by phosphorimaging of APPs after biosynthetic labelling of the lymphoblastoid cell lines with ^{35}S methionine followed by a 2 hour chase and immunoprecipitation of the cell lysates with an antibody specific for the C-terminus of APP. Lane 1 represents APP analysis of the affected individual. Lanes 3 and 4 represent control cell lines. Lane 5 represents APP analysis of the affected individual's "at risk" son. Lane 6 represents APP analysis of the affected individual's "at risk" brother. Lane 7 represents a third control cell line.

Figure 3 is a photographic representation of an electrophoretic gel showing a low molecular weight form of 100 kD in all the control cell lines after 5 hours of biosynthetic labelling (Fig. 3A, lanes 5-10) and the 130-140 kD secreted APP in the affected patient with the APP717 mutation (Fig. 3A, lanes 3,4). The antibody used in this study was specific for the first 16 residues of A β . The "at risk" individual (Fig 3A, lanes 1,2) and an FAD individual with a defect on chromosome 14 (Fig 3B, lanes 1, 2) also exhibited the longer molecular form of secreted APP.

Figure 4 is a photographic representation of an electrophoretic gel showing that labelling with ^{35}S methionine for a total of 14 hours results in an even more marked increase in the 130-140 kD secreted APP for the affected patient with the APP717 mutation (Fig.4A & Fig. 4B, lane 1) as well as the "at risk" individuals carrying the APP717 mutation (Fig. 4A & Fig. 4B, lanes 4,5).

30 Examples

Reagents - Monoclonal antibodies 6E10 and 4G8 were purchased from Drs. Kim and Wisniewski (Institute for Basic Research, Staten Island). Their preparation is described in Kim, K.S., Wen, G.Y., Bancher, C., Chen, C.J.M., Sapienza, V.J., Hong, H. and Wisniewski, H.M. (1990) NeuroSci. Res. Commun. 2, 121-130. Affinity-purified rabbit-C-terminal APP antibody termed 369 was produced according to the method in Buxbaum, J.D., Gandy, S.E., Cicchetti, P., Ehrlick, M.E., Czernik, A.J., Fracasso, R.P., Ramabhadran, T.V., Unterbeck, A.J. & Greengard, P. (1990) Proc. Natl. Acad. Sci. USA 87, 6003-6006. Gamma Bind Plus Sepharose and Protein A-Sepharose CL-4B was obtained from Pharmacia LKB. 10-20% Tris/ tricine gradient gels were purchased from Novex (San Diego, CA). [³⁵S] methionine was purchased from Australian Biosearch (Perth, WA).

Cell Lines - Early onset FAD lymphoblast cell line with a mutation in the PS1 gene were obtained from the Coriell Cell Repositories, Coriell Institute for Medical Research, Camden N.J.

Lymphoblastoid cell lines were established, from a recently identified early onset Australian FAD pedigree with a mutation on codon 717 (Brooks, W.S., Martins, R.N., De Voecht, J., Nicholson, G.A., Schofield, P.R., Kwok, J.B., Fisher, C., Yeung, L.U. and Van Broeckhoven C. (1995) Neurosci. Lett. 199, 1-4.). Cell lines were immortalised by infecting with Epstein Barr virus employing standard procedures (Ventura, M., Gibaud, A., Le Pendu, J., Hillaire, D., Gerard, G., Vitrac, D. and Orbiol, R. (1988). Hum. Hered. 38, 36-43.). Cell lines used in this study were obtained from an affected individual, her spouse, her "at risk" son and brother, her normal mutation-free son and sister and an unrelated normal control with no family history of the disease.

Biosynthetic Labelling - Cells were pelleted by centrifugation for 5 minutes at 200 x g. The media was aspirated and the cells resuspended in Hanks Balanced Salts (HBS) solution and centrifuged again as described above. The cells were resuspended again in HBS and centrifuged once more for 5 minutes at 200 x g. HBS was replaced with methionine free medium and the cells

- 13 -

incubated for 45 minutes at 37°C. The cells were then incubated with 250 μ Ci (9.75 MBq) [35 S] methionine in 1 ml of methionine-free DMEM. Metabolic labelling was carried out for either 40 minutes, 5 hours or 16 hours at 37°C. The 40 minute pulse was followed by a chase period of 2 hours in media
5 containing 200 mM unlabelled methionine. After the 2 hour chase, the cells were centrifuged as described above and washed twice with HBS. After the second wash, the cell pellet was solubilised in lysis buffer (50 mM TrisCl pH7.4 containing 150 mM NaCl, 0.5% NP40, 0.5% Sodium deoxycholate, 0.25% SDS, 5 mM EDTA, 50 mg/ml leupeptin, 0.25 mM PMSF and 10 mg/ml aprotinin). The
10 solubilised lysate was centrifuged at 10,000 x g for 5 minutes. The supernatant's were removed and normalised for total protein by diluting the high protein samples in lysis buffer.

Immunoprecipitation - The supernatant's were incubated at 4°C with monoclonal antibodies 6E10/4G8 or other antibodies directed at similar epitopes
15 of the A β molecule overnight for media or with the polyclonal antibody 369 or similar antibodies directed against the C-terminus of APP for lysates. Immune complexes were immunoprecipitated with Gamma Bind Plus Sepharose or with Protein A Sepharose for the monoclonal and polyclonal antibodies respectively. The immune complexes were washed with 1 ml of 0.5M NaCl in Tris-buffered
20 saline (100 mM Tris-HCl, 150 mM NaCl, pH 7.4) for 1 hour at 4°C. The pellets were resuspended in 0.1 ml of wash buffer and the suspension was applied to a 1 ml sucrose cushion and centrifuged at 10,000 x g for ten minutes. Samples were boiled in 20 ml of Tris/tricine sample buffer and separated on 10-20% Tris/Tricine gradient polyacrylamide gels. Gels were treated for fluorography
25 with enhancer solution (Entensify; New England Nuclear-Dupont, Boston MA), dried, and quantitated with a Phosphorimager (Fuji).

Protein Quantitation - The concentration of total protein in each lysate sample was estimated using a protein assay, as per the manufacturers' instructions (BSA Protein Assay Kit, Pierce (Rockford, IL, USA)).

A family tree depicting members of an Australian family with a mutation in the APP gene at codon 717 where the amino acid valine is substituted with isoleucine is shown in Figure 1. Those family members whose lymphocytes were immortalised by infection with Epstein-Barr virus and used for the study of APP metabolism are indicated by an associated asterisk (Fig. 1).

Biosynthetic labelling of the lymphoblastoid cell lines with ^{35}S methionine followed by a 2 hour chase and immunoprecipitation of the cell lysates with antibody #369 was carried out followed by visualisation by phosphorimaging of APPs (Fig 2). The three control cell lines in lanes 3,4 and 7 exhibited the classical profile, as seen for other cell types, of an immature 100 kD form and a mature 110 kD form of APP. The affected individual in lane 1 (Fig.2) exhibited a number of APP isoforms in addition to the immature and mature forms observed in the controls. A prominent 130 to 140 kD protein was observed in the affected patient's lymphoblastoid cell line (lane1) and was also observed in the affected's "at risk" son (lane 5; Fig.2) and "at risk" brother (lane 6; Fig.2). The "at risk" son had abundant levels of a protein with a molecular weight between 100 kD and 110 kD (lane 6; Fig. 2). Taken together, the APP lysate profiles for the controls differ significantly from those carrying the APP mutation at codon 717 confirming that APP processing is impaired in the latter group.

B-lymphocyte derived cell lines with mutations in the APP gene at codon 717 and a cell line with a defect linked to chromosome 14 secrete abundant levels of high molecular weight APP ranging in size from 130 to 140 kD (Fig.3 and Fig.4) in the absence of exogenous stimulation. These high molecular forms of APP are not observed in control lymphoblastoid cells (Fig. 3 and Fig.4). A lower molecular weight form of 100 kD was observed in all the control cell lines after 5 hours of biosynthetic labelling (Fig. 3A, lanes 5-10) and in the affected patient with the APP717 mutation (Fig. 3A, lanes 3,4). Further labelling with ^{35}S methionine for a total of 14 hours resulted in an even more marked increase in the 130-140 kD secreted APP for the affected patient with the APP717 mutation (Fig.4A & Fig. 4B, lane 1) as well as the "at risk" individuals carrying the

APP717 mutation (Fig. 4A & Fig. 4B, lanes 4,5). This data shows that abundant levels of higher molecular weight secreted APP are observed in cell lines obtained from early onset FAD patients with the APP 717 mutation as well as the early onset FAD cell line lacking a mutation in the APP gene but with a defect
5 linked to chromosome 14 (cell line AM12618).

Matsumoto and Fujiwara (1994) Biochemistry 33, 3941-3948 were the first to report that lymphoblastoid cell lines from FAD patients exhibited abnormal processing of APP. These authors identified a 120 kD complex only in FAD conditioned medium which on boiling broke down to 53 kD and 65 kD APP
10 fragments and a 70 kD protein. The 70 kD protein was subsequently identified as an FAD associated Ca^{2+} dependent protease which cleaves the N-Terminus of $\text{A}\beta$ (Matsumoto, A. and Fujiwara, Y. (1994) Biochemistry 33, 3941-3948.). In contrast, the present method employs a specific APP antibody, that recognises the first 16 amino acid residues of $\text{A}\beta$, to establish that early onset FAD
15 lymphoblastoid cells secrete abundant levels of a 130 -140 kD form of APP. The latter observation was extended to cell lines from individuals "at risk" but who were below the age of onset.

The CLAIMS defining the invention are:

1. A method for detecting Alzheimer's disease in a patient said method comprising the steps of:
 - 5 (i) selecting from a patient, suspected of having Alzheimer's disease, a fluid sample containing secreted amyloid precursor protein;
 - (ii) reacting the fluid sample proteins with a binding substance specific for either the β -amyloid peptide or the amyloid precursor protein or both; and
 - 10 (iii) assaying for the presence of a higher than normal quantity of secreted amyloid precursor protein with an average molecular weight of greater than about 130 kilodaltons, compared to patients negative for Alzheimer's disease.
- 15 2. A method according to claim 1 wherein the fluid sample is selected from blood plasma or cerebral spinal fluid.
3. A method for detecting Alzheimer's disease in a human said method comprising the steps of:
 - 20 (i) selecting a biological sample from a patient suspected of having alzheimer's disease, wherein said sample is isolated from a cell source which in alzheimer's disease patients is known to produce secreted amyloid precursor protein;
 - (ii) growing the biological sample selected in step (i) under suitable cell culture conditions for sufficient time to allow amyloid precursor protein expression and secretion into the culture supernatant;
 - 25 (iii) reacting, the culture supernatant proteins, with a binding substance specific for either the β -amyloid peptide or the amyloid precursor protein or both; and
 - (iv) assaying the proteins selected in step (iii) for the presence of a higher than normal quantity of protein with an average molecular

weight of greater than about 130 kilodaltons, compared to patients negative for Alzheimer's disease.

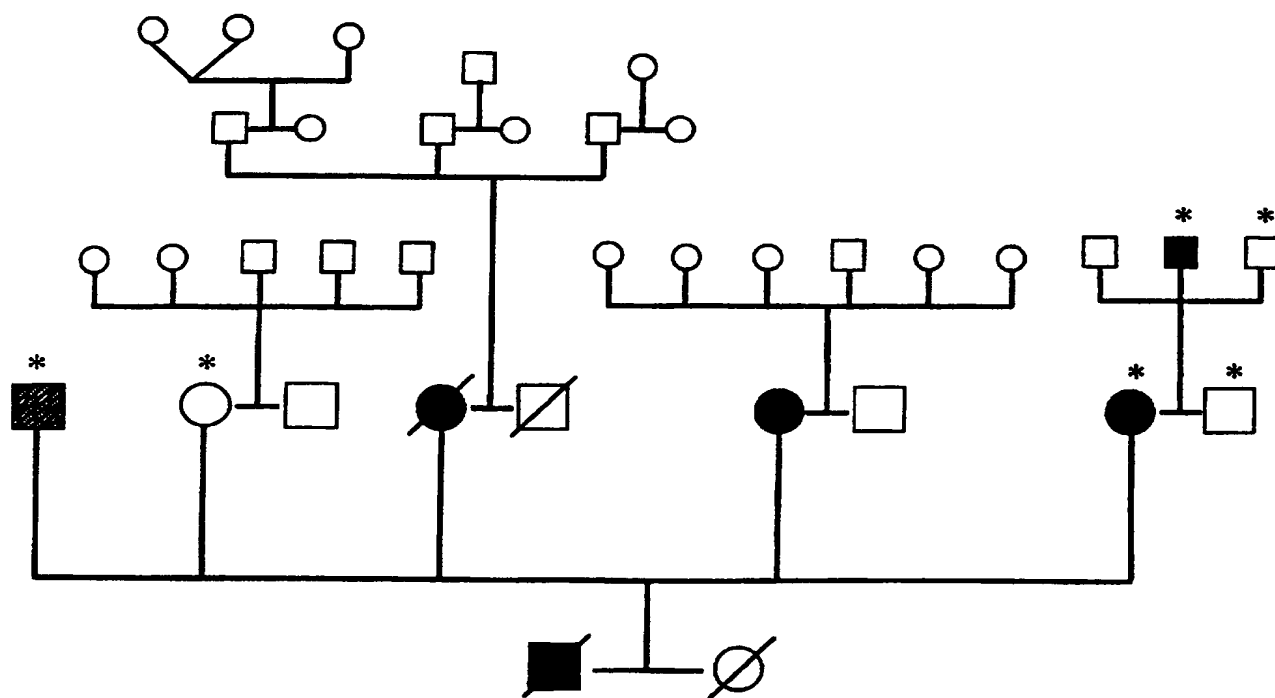
4. A method according to claim 3 wherein the biological sample is selected from lymphoblastoid cells or skin fibroblasts.
- 5 5. A method according to claim 4 wherein the biological sample is lymphoblastoid cells.
6. A method for detecting Alzheimer's disease in a human said method comprising the steps of:
 - 10 (i) selecting a lymphoblastoid cells from a patient suspected of having alzheimer's disease;
 - (ii) growing the lymphoblastoid cells in cell culture in the presence of a labelled amino acid under suitable conditions for sufficient time to allow amyloid precursor protein expression and secretion into the culture supernatant;
 - 15 (iii) separating, at a suitable time after incubation, the culture supernatant from the cells;
 - (iv) disrupting the cells to release all of their cytoplasmic and membrane proteins;
 - 20 (iii) reacting separately the cell lysate and culture supernatant proteins, with a binding substance specific for either the β -amyloid peptide or the amyloid precursor protein or both; and
 - 25 (iv) assaying the proteins selected in step (iii) for the presence of a higher than normal quantity of protein with an average molecular weight of greater than about 130 kilodaltons, compared to patients negative for Alzheimer's disease.
7. A method according to anyone of the preceding claims wherein the APPs selected by the binding substance have an average molecular weight of about 130 to 140 kD.

- 18 -

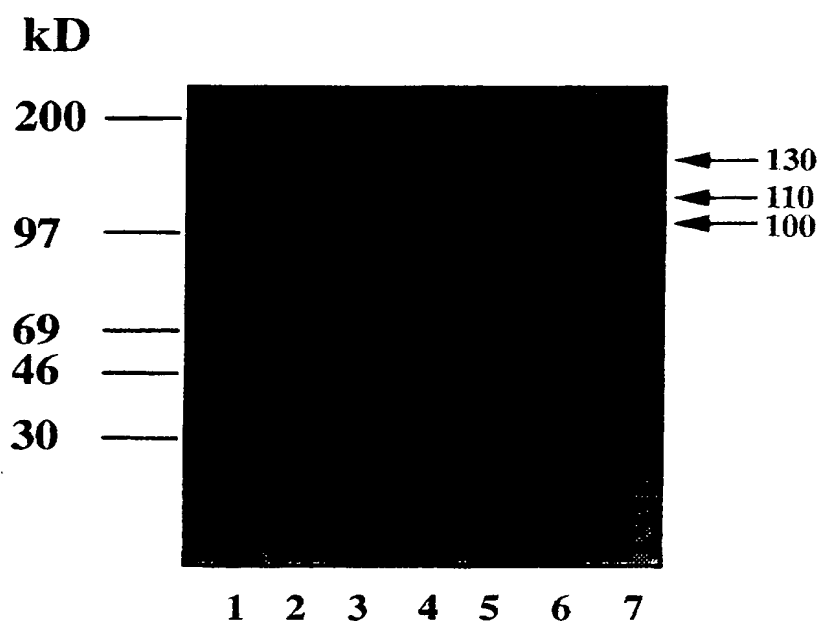
8. A method according to anyone of the preceding claims wherein the binding substance(s) are selected from the group consisting of: antibodies, antibody fragments or antibody complexes which recognise at least a epitope within the A β peptide and/or the C-terminus of the APP.
- 5 9. A method according to claim 8 wherein the binding substance is either monoclonal or polyclonal antibodies.
10. A method according to anyone of the preceding claims wherein the binding substance recognises part or all of one or more of the following sequences: amino acids 17 through 24 of the A β peptide, amino acids 1 through 16 of
10 the A β peptide, or the C-terminus of amyloid precursor protein.
11. A method according to any one of the preceding claims wherein the binding substance is labelled.
12. A method according to anyone of the preceding claims wherein the proteins
15 are precipitated and concentrated and then incubated with the binding substance.
13. A method according to any one of claims 3 to 6 wherein the cells are immortalised after selection.
14. A method according to any one of claims 3 to 6 wherein immortalisation is achieved by fusing the cells to a stabilised cell line.
- 20 15. A method according to any one of claims 3 to 6 wherein immortalisation is achieved by infecting the cells with a transforming virus.
16. A method according to claim 15 wherein the transforming virus is Sendai virus or Epstein Barr virus.
17. A method according to claim 13 wherein immortalisation is achieved by
25 transfecting the lymphoblastoid cells with transforming genes to create permanent or semipermanent cell lines.

18. A method according to either of claims 5 or 6 wherein the lymphoblastoid cells are incubated in RPMI 1640 medium containing 5 to 30% foetal calf serum at about 30 to 40°C in the presence of 1 to 10% CO₂ to enable sufficient APP production.
- 5 19. A method according to either of claims 5 or 6 wherein the lymphoblastoid cells are incubated in RPMI 1640 medium containing 20% foetal calf serum at 37°C in the presence of 5% CO₂ to enable sufficient APP production.
20. A method according to either of claims 5 or 6 wherein the lymphoblastoid cells are incubated in the presence of a radioactive amino acid.
- 10 21. A kit for detecting Alzheimer's disease in a patient, said kit comprising: (i) a binding substance specific for either the β -amyloid peptide or the amyloid precursor protein or both; and (ii) instructions consistent with the method of anyone of the preceding claims.
- 15 22. A kit according to claim 21 wherein the binding substance(s) are selected from the group consisting of: antibodies, antibody fragments or antibody complexes which recognise at least a epitope within the A β peptide and/or the C-terminus of the APP.
23. A kit according to claim 21 wherein the antibodies are either monoclonal or polyclonal antibodies.
- 20 24. A kit according to claim 21 wherein the binding substance(s) recognises part or all of one or more of the following sequences: amino acids 17 through 24 of the A β peptide, amino acids 1 through 16 of the A β peptide, or the C-terminus of amyloid precursor protein.
- 25 25. A method for detecting Alzheimer's disease in a patient substantially as described with particular reference to the examples.

FIGURE 1

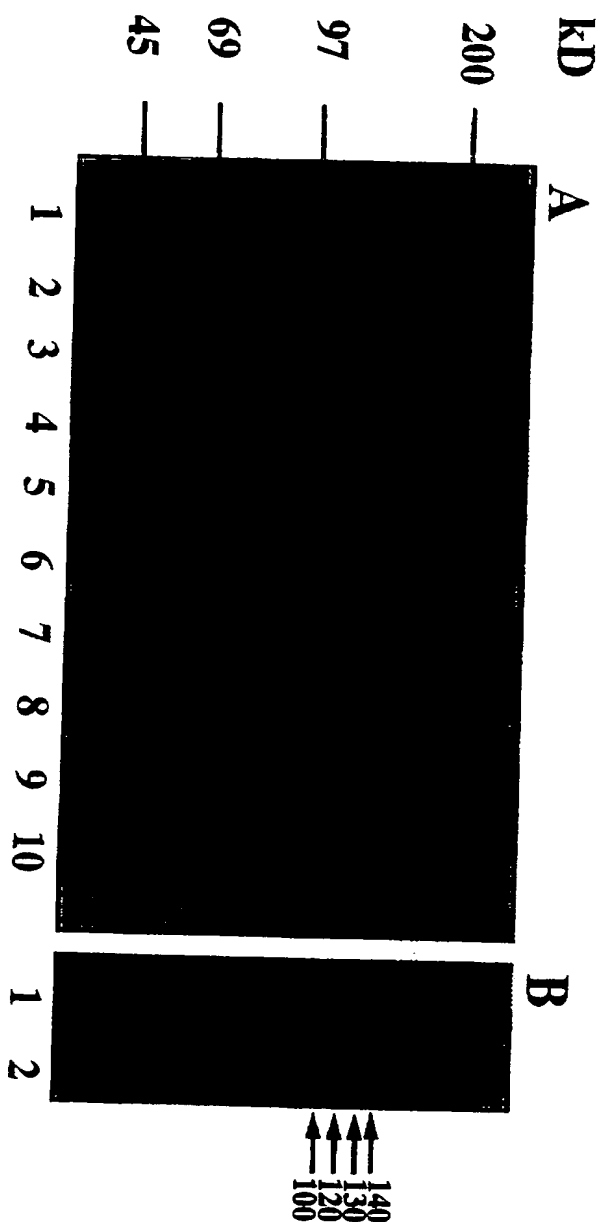


2/4
FIGURE 2

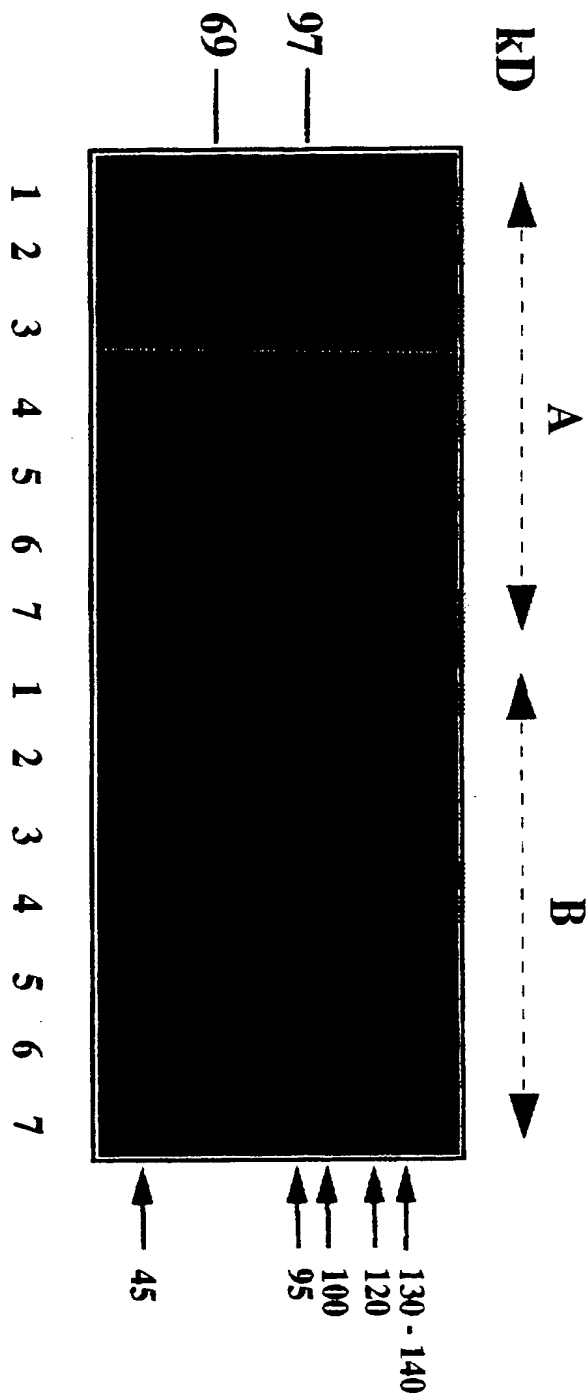


3/4

FIGURE 3



4/4
FIGURE 4



INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 96/00711

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl⁶: G01N 33/68, 33/539

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC : G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

AU : IPC as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DERWENT : [Amyloid () Precursor or APP or APP695 or APP751 or APP770] and Alzheimer:

CHEMICAL ABSTRACTS : (File CA): [Amyloid () Precursor? Or APP695 or APP751 or App770] and [Alzheimer? (S) (Screen? Or Diag?)]

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 90/15331 A (E.I. DUPONT DE NEMOURS AND COMPANY) 13 December 1990 entire document	1-25
X	WO 93/10459 A (THE UNIVERSITY OF MELBOURNE) 27 May 1993 entire document	1-25



Further documents are listed in the continuation of Box C



See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
 "E" earlier document but published on or after the international filing date
 "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
 "O" document referring to an oral disclosure, use, exhibition or other means
 "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
 "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
 "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
 "&" document member of the same patent family

Date of the actual completion of the international search. . . .
 17 December 1996

Date of mailing of the international search report

7 JAN 1997

Name and mailing address of the ISA/AU
 AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION
 PO BOX 200
 WODEN ACT 2606
 AUSTRALIA Facsimile No.: (06) 285 3929

Authorized officer

T. SUMMERS

Telephone No.: (06) 283 2291

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/AU 96/00711

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	Buxbaum J.D. et al., 'Processing of Alzheimer β /A4 amyloid precursor protein: modulation by agents that regulate protein phosphorylation', (1990) <i>Proc. Natl. Acad. Sci. USA</i> 87, 6003-6 page 6003 column 2 second paragraph under 'materials and methods' entire document	21-24 1-20, 25
X A	Selkoe D.J. et al., ' β -Amyloid precursor protein of Alzheimer disease occurs as 110- to 135-kilodalton membrane-associated proteins in neural and nonneural tissues' (1988) <i>Proc. Natl. Acad. Sci. USA</i> 85, 7341-5 page 7341 column 2 paragraph preparation of antibodies to synthetic peptides entire document	21-24 1-20, 25
X	Kim K.S. et al., 'Production and characterisation of monoclonal antibodies reactive to synthetic cerebrovascular amyloid protein' (1988) <i>Neuro Sci. Res. Commun.</i> , 2(3) 121-30 abstract, Table 1	21-24
X A	Games D. et al., 'Alzheimer type neuropathology in transgenic mice overexpressing V717F β -amyloid precursor protein', (9 February 1995) <i>Nature</i> 373, 523-7 $A\beta$ and APP antibodies in Figs 1-3 entire document	21-24 1-20, 25
X A	Matsumoto A. et al., 'Abnormal and deficient processing of β -amyloid precursor protein in familial alzheimer's disease lymphoblastoid cells', (1991) <i>Biochem. Biophys. Res. Commun.</i> 175(2), 361-5 page 362 under 'Antibodies' entire document	21-24 1-20, 25
X A	Suzuki N. et al., 'An increased percentage of long amyloid β protein secreted in familial amyloid β protein precursor (β APP 717) mutants' (1994) <i>Science</i> 264, 1336-40 page 1337, column 1 last paragraph - column 2 entire document	21-24 1-20, 25
X	WO 94/05811 A (BRISTOL-MYERS SQUIBB COMPANY et al.) 17 March 1994 claims 28-30, pages 24-25	21-24
X	Pardridge W.M. et al., 'High molecular weight alzheimer's disease amyloid peptide immunoreactivity in human serum and CSF is an immunoglobulin G' (1987) <i>Biochem. Biophys. Res. Commun.</i> 145 (1), 241-8 abstract	21-24
A	WO 92/13069 A (IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY & MEDICINE) 6 August 1992 entire document	1-25
A	WO 93/07296 A (INDIANA UNIVERSITY FOUNDATION) 15 April 1993 entire document	1-25

Information on patent family members

PCT/AU 96/00711

Patent Document Cited in Search Report				Patent Family Member			
WO	9015331	AU	58173/90	EP	474765	US	5234814
WO	9310459	AU	29263/92	EP	613560		
WO	9405811	EP NO	659217 950778	FI	950883	HU	70456
WO	9213069	AU	11694/92	EP	568575		
WO	9307296	AU	27659/92				